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# Side Effects of Human Drug Use: An Overview of the Consequences of Eels' Exposure to Cocaine

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**Abstract:** The widespread use of drugs is a global problem which affects not only humans but also the environment around them, as research is showing the presence of these substances in different environmental matrices, like air, water, and soil. Above all, due to the remarkable pharmacological properties of drugs, it is discovered that organisms accidentally exposed to them, as aquatic organisms, undergo behavioral and physiological changes that can compromise their health, survival, and reproduction ability. In addition to this, we must consider the ability of some drugs to accumulate within these organisms, thus entering the food chain, and the possible interactions that drugs in water can establish with each other and with other possible pollutants, making the final effects on exposed organisms unpredictable. This article is an overview of the effects of one of these drugs, cocaine, one of the drugs commonly found in the aquatic environment, on European eel, an endangered species and known biomonitor of aquatic contamination.

**Keywords:** *Anguilla anguilla*; cocaine; endocrine system; histopathological changes; illicit drugs; neuroendocrine effects; water pollution



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## 1. Introduction

Illicit drug use is a growing phenomenon, which, as numerous studies have shown, poses a threat not only to human health but also to the environment. In 2020, an estimated 284 million people worldwide, aged 15–64, had used a drug of abuse within the last 12 months; of these users, approximately 13.6 per cent are estimated to suffer from drug use disorders [1]. At the same time, the environmental impact of the drugs of abuse appears increasingly evident; cultivation and production of plant-based and synthetic drugs, and drug use, have many consequences like energy use, deforestation, soil and water pollution and depletion, air pollution, food chain effects and biodiversity loss. Although the global effects of the activities related to the cultivation and production of illicit drugs are less significant than those of the pharmaceutical industry and agriculture, they can be important at local or community level [2].

Cocaine, for example, is estimated to have been consumed at least once by about 21.5 million people (0.4 percent of the global population aged 15–64) in 2020 [1]. It is estimated that 1982 tons of pure cocaine were produced in 2020, an increase of 11% over the previous year. The carbon footprint (a measure that expresses the total emissions of

greenhouse gases, represented as carbon equivalents) of cocaine, related to cultivation of coca plants, processing of cocaine, disposal of waste generated in the manufacturing process and land-use change, is 4500 kg $CO_2e$ per kg of cocaine produced. Therefore, referring to the 2020 data, we obtain a mean value of the total emissions per year of 1.9 million tons of $CO_2e$, a value significantly higher than that of other crops, as sugar cane or cocoa beans [2].

Another important environmental effect of drugs of abuse, as well as human and veterinary medicines, is their ability to contaminate the aquatic environment and influence the organisms living there [3–7]. Indeed, the intake of drugs of abuse is followed by their metabolization, usually partial, and the excretion of the parent drug and its metabolites especially by the renal way, that is the primary way in which these substances are removed from the body, though they can also be excreted through stool, sweat, tears and saliva [8]. Moreover, another possible source of contamination is the occasional discharge of drugs of abuse by clandestine laboratory wastes into sewage systems [3]. Therefore, illicit drugs and their metabolites reach treatment plants that do not always remove these substances, mainly because they are not always designed to do so. As a result, both drugs of abuse and their metabolites can be found in treated wastewater effluents and surface water, groundwater and drinking water [9–14]. Although the concentrations of drugs of abuse and their metabolites, found in surface waters around the world, are rather low (between $ng \cdot L^{-1}$ and $\mu g \cdot L^{-1}$), the continuous exposure and the remarkable pharmacological properties of drugs of abuse and their metabolites, sometimes greater than the parent drug, raise concerns about the fate of aquatic species living in contaminated sites. Indeed, scientific evidence is increasingly showing that different animal and plant aquatic organisms can bioaccumulate the different drugs of abuse and their metabolites, often suffering toxic effects [4–7]. In this paper, particular consideration will be given to cocaine present in the aquatic environment, and its effects on aquatic fauna and especially on European eels.

## 2. Cocaine

In nature, cocaine is present as an alkaloid in plants belonging to the Erythroxylaceae family, and in greater quantities in *Eritroxylum coca* and *Eritroxylum novogranatense*, two shrubs that grow spontaneously in South America. Cocaine can exist in several formulations; the predominantly used, and historically best known, cocaine formulation is the hydrochloride form. It is highly soluble in water and taken mainly intranasally (snorted), but may also be injected (subcutaneously, intramuscularly, or intravenously) and swallowed. The intranasal route is the most frequently used by regular users and ensures a quick onset of effects due to the high vascularization of the nasal mucosa. The subcutaneous and intramuscular pathway, due to the vasoconstrictor effect, involve slower absorption and therefore the effects are less rapid than the intravenous route. Intravenous bioavailability is 100%. Another widespread form is cocaine free base (basic form of cocaine hydrochloride), which is the transformation of water-soluble cocaine into the alkaloid base. It is mostly smoked but can also be injected; the effects appear within 5–10 s giving a very short "high" but very intense. Given the remarkable absorption surface of pulmonary alveoli, the inhalation route ensures the absorption of doses particularly high in the short term, which may explain the danger of cocaine in this form. It is relatively ineffective when administered intranasally or in a vein. Finally, "crack" cocaine is the name given to cocaine crystals obtained by processing cocaine to turn it into a substance that you can smoke. It's mostly smoked or injected, but it can also be ingested; its effects are like freebase [8]. Cocaine production is mainly concentrated in the Andean area of South America with the three largest producing countries (Colombia, Peru, and Bolivia), as *Erythroxylon coca* grows spontaneously in the warm and humid tropical climates of South America at an altitude of between 700 and 2000 metres. The neighbouring countries (Brazil, Venezuela, Argentina, and the Caribbean) play an important role as storage areas and transit zones for exports to Europe and the United States of America [2].

Cocaine belongs to the class of psychomotor stimulants, a group of drugs including nicotine, caffeine, and related substances, also known as minor stimulants, and am-

phetamines, cocaine, methylphenidate, and several dopamine agonists, also known as major stimulants. These stimulants, at low to moderate doses, increase vigilance, resistance to sleep and alertness; within a certain dose range, they also increase the performance in different tasks and locomotor activity, due to their sympathomimetic effects, as increased blood pressure and heart rate, constriction of blood vessels to the viscera, increased body temperature and muscle tension, and bronchial dilation. However, also direct adverse consequences on the body can be observed, as in the case of cocaine, which can cause neurological and cardiovascular problems like cerebral hemorrhage, convulsion induction, lethal cardiac arrhythmias, and acute myocardial infarction, even at low doses [8].

The main effect of cocaine on the nervous system stems from its ability to bind to monoamine transporters on nerve endings: serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET). This binding inhibits the reuptake of monoamines from the synaptic cleft, where the concentration of monoamines, and the consequent stimulation of the respective receptors, is increased. Although the effect of cocaine is exerted on all monoamines, the reinforcing properties of cocaine are mainly due to its effects on dopamine (DA), the extracellular levels of which increase dramatically following chronic cocaine exposure [15]. This increase, however, is followed by a rapid return to normal DA levels, for the activation of homeostatic mechanisms such as the activation of DA autoreceptors, which causes the user to increase the dose of cocaine administered. In time, the users come to the depletion of intraneuronal DA, due to different mechanisms: reduced DA synthesis via autoreceptor activity; enhanced extraneuronal catabolization via catecholamine o-methyl transferase (COMT) enzyme, or intraneuronal catabolization via monoamine oxidase (MAO) enzyme; decreased postsynaptic DA receptor availability due to excessive and prolonged exposure to DA. All these processes result in decreased dopaminergic activity with chronic cocaine use and contribute to cocaine dependence [8].

While the rewarding effects of cocaine are basically linked to DAT transporters, cocaine toxicity is especially related to both DAT and SERT transporters. In addition, cocaine is also able to bind to sigma and muscarinic acetylcholine receptors. Sigma receptors can be divided into two subtypes: sigma-1 and sigma-2; these receptors contribute to the toxicity of cocaine although it is not known which of the two subtypes is involved. As regards muscarinic receptors, cocaine probably behaves as a partial agonist, but the exact mechanism of action is not known. Cocaine can also bind to different voltage-gated ion channels, a binding responsible for the properties of local anesthetic of cocaine and for cocaine cardiotoxicity. Moreover, cocaine can bind, in a pH dependent manner, to two serum proteins, involved in cocaine toxicity: albumin and alpha 1 acid glycoprotein; the extent of binding to these proteins affects the concentration of cocaine free to exert its effects. Although the toxic effects of cocaine are mainly related to its binding with monoamine transporters, sigma and muscarinic acetylcholine receptors and cardiac ion channels, it is believed that two other neurotransmitters, gamma-aminobutyric acid (GABA) and glutamate (GLU), the most common inhibitory and excitatory neurotransmitters, respectively, may be involved in them [15].

After intake, cocaine is distributed throughout the body and is rapidly metabolized by different enzymatic pathways and non-enzymatic hydrolysis, also according to route of administration [16,17] in several metabolites, as benzoylcgonine, ecgonine methyl ester and norcocaine, the latter responsible for the toxic effects on the liver. A small amount of cocaine is excreted intact in the urine; the half-life of cocaine is between 30 and 90 min, whereas that of its metabolites is longer.

Following the excretion of cocaine and its metabolites with the urine, and occasional discharge of drugs of abuse by clandestine laboratory wastes into sewage systems [3], cocaine and its metabolites reach fresh and marine surface waters, where cocaine can be found in concentrations ranging from about 0.13 ng $L^{-1}$ to 5896 ng $L^{-1}$ in freshwaters [18–20] and from 2.4 ng $L^{-1}$ to 537 ng $L^{-1}$ in coastal zones [10,21]. The pharmacological characteristics

of cocaine, and continuous exposure to it, make this substance potentially harmful to fish living in contaminated waters, such as eels.

## 3. European Eel

The European eel (*Anguilla anguilla*) is a migratory, catadromous fish species, consisting of a single stock, distributed throughout the European continent as well as in the Mediterranean basin, which reproduces in the Atlantic Ocean and for which the panmixia hypothesis is currently accepted. Eel fishing is carried out throughout the distribution area of the species and concerns the juvenile and preadult stages, but the conservation of the stock depends on the recruitment and emigration of breeding animals at sea. Eel farming is practiced in many countries, for a European level of around 8000 tons; the production completely depends on the wild seed, since the reproduction artificial, although implemented experimentally, does not go beyond the larval stage, at least in the European eel. Adult eels can survive in both air and water thanks to the fact that respiratory exchanges can occur both through the gills and through the skin. Eels are present in a wide range of aquatic habitats (rivers, canals, estuaries, lakes, ponds, and lagoons), in relation to their great adaptability to different environmental conditions; moreover, being a euryhaline species, eels can adapt to both fresh and sea water, and well tolerate variations in oxygen concentrations. As a primarily bottom-dwelling fish, the eels rely in their feeding on the prey population that is present there and prefer to eat at night [22,23].

The biological cycle of the eel is considered unique in relation to the nature and extent of reproductive migration. This life cycle takes place mainly in continental fresh waters, for most of the life of the eel, and in open ocean during the reproductive phase. Moreover, the life cycle involves a succession of metamorphic stages: leptocephalus larvae, hatching from eggs in the Sargasso Sea and heading for continental waters; glass eels that move into continental waters; yellow eels that remain in continental waters until sexual maturity is reached; silver eels that migrate back to reproductive areas [22,23].

This complex life cycle exposes the eels to different and numerous stressors, and many studies have shown that overfishing, habitat loss, pest attack and water contamination pose a serious threat to eel survival. In particular, the presence of fat in the eels, their long stay in the same area and their ability to accumulate contaminants especially lipophilic, make this species particularly susceptible to aquatic contamination. Indeed, to date, the European eel is included, according to IUCN, among the endangered species and considered at risk of extinction [22,24–26]. In addition to the different types of contaminants present in the water, illicit drugs are proving to be a new, widespread class of contaminants with remarkable pharmacological properties, that are likely to have effects on aquatic fauna [3–7].

Therefore, our research group has begun to study the effects of one of these illicit drugs, cocaine, on European eel, to understand how this substance could affect the physiology and reproductive capacity of this species. The experiments were carried out on silver eels, which, after a month's acclimatization, were exposed to an environmental concentration of cocaine (20 ng/$L^{-1}$), among those measured in surface water. The eels were housed in aquariums with a capacity of 300 L, in dechlorinated and well-aerated tap water, exposed to natural photoperiod and not fed, as in the silver stage eels do not normally feed. Moreover, the following parameters of the water were established and monitored: dissolved oxygen 8.1 ± 0.5 mg/L; salinity 0, temperature 15 ± 1 °C, pH 7.3 ± 0.2, ammonia < 0.1 mg/L. For the exposure, a stock solution of 3 mg/500 mL of cocaine free-base in ethanol was prepared and kept in the refrigerator. During the exposure, 1 mL of the stock solution was administered every day, after the change of the water, directly in each aquarium for 30–50 days. At the same time control groups, exposed to tap water only, and carrier groups, exposed to ethanol only, in the same concentrations as eels receiving cocaine, were set up. At the end of exposure, some of the exposed eels were deprived of cocaine and exposed to tap water only, for 3–10 days, to verify their recovery ability. At the end of the exposure, or the recovery period, histological, histochemical, biochemical, and molecular biology analyses were performed [27]. All these studies were carried out in accordance with EU

Directive 2010/63/EU for animal experimentation and institutional guidelines for care and use of laboratory animals and were authorized by the Italian Ministry of Health's General Directorate of Animal Health and Veterinary Drugs.

## 4. The Effects of Cocaine on the European Eel

### *4.1. Accumulation of Cocaine in Eel Tissues*

Cocaine is able to accumulate in biological tissues. From early studies, which showed the presence of cocaine in tissues of victims of drug-related deaths [28] and in rats exposed to cocaine [29], there is increasing evidence that aquatic organisms can accumulate cocaine [6,30–32] and its metabolites, for example benzoylecgonine [33], present in the aquatic environment. Consistent with literature data, eels chronically exposed to cocaine accumulated this drug in almost all their tissues, albeit to a different degree, with highest concentrations in brain, muscle, liver and kidney; lower concentrations in digestive tract, gills and skin, and lowest concentrations in spleen and gonads. After a recovery period of three days, cocaine was still present, but at much lower concentrations, indicating that recovery is possible, but takes longer [34]. The differential accumulation of cocaine in different organs of the eel agrees with the tissue-specific accumulation of methamphetamine and ketamine in zebrafish (*Danio rerio*) [35], and antidepressants [36] in several species of fish. The presence of high levels of cocaine in the brain of the eel reflects the affinity of this drug for nervous tissue and agrees with the accumulation of antidepressants in brain of white suckers [37] and fathead minnows [38]. The presence of high levels of cocaine in muscle can be explained by the fat solubility of cocaine and its tendency to deposit in adipose tissue [29], of which the eel muscle is rich (35.06%) [39]. In contrast, the low methamphetamine and ketamine level found in the muscle of zebrafish may depend on the low lipid content typical of the lean fish group, to which zebrafish belongs [40]. Finally, liver and kidney are involved in drug metabolization, and this may explain the high level of cocaine accumulation found in eel. Digestive tract, skin and gills, the first interfaces exposed to the water and its pollutants, represent the main routes of entry of cocaine, since the eels during the exposure were not feed, and showed low levels of cocaine, whereas spleen and gonads had the lowest levels, a result in accordance with what was observed for other drugs [35,36] and perhaps related to a poor metabolization capacity.

### *4.2. Neuroendocrine Effects of Cocaine in the Eels*

#### 4.2.1. Nervous Tissue

It is well known that main effects of cocaine stem from its ability to bind to DAT transporters and increase DA concentration in the synaptic cleft [16]. In fish, DA plays a key role in nervous system physiology, regulating numerous activities as hypothalamic and pituitary functions, locomotor activity, thermoregulation, and food intake. Moreover, in eels, DA was found to be involved (1) in sexual maturation, since it inhibits the synthesis and release of gonadotropins, and gonadal development, and (2) in last steps of eel reproduction and in reproductive migration [41,42]. Therefore, a variation in the levels of cerebral DA could seriously affect the physiology of this species. Our results showed that eels chronically exposed to cocaine had increased brain DA levels, which increased even more in recovery specimens [43]. This observation agrees with the increases in brain dopamine content found in *Danio rerio* after 72 h withdrawal from repeated cocaine administration, probably due to overall decrease in the expression of mRNA for DA transporter [44], a hypothesis that could also be valid for eels.

Cocaine is also a sympathomimetic drug, that facilitates norepinephrine (NE) transmission, and produces autonomic effects that reflect increases in sympathetic activity such as increased heart rate or blood pressure or dilation of bronchioles in the lungs [8]. Many studies, indeed, showed that cocaine activates the sympathoadrenal system, increasing the levels of plasma catecholamines (CA): NE and epinephrine (E) produced by the adrenal chromaffin cells [45]. Consistent with this data, eels chronically exposed to cocaine, and even more recovery eels, showed elevated plasma levels of DA, NE, and E [43]. It is

well known that, in fish, physiological functions during stress are mainly regulated by the humoral release of CA, which also perform key functions such as increase in glucose synthesis and lipid metabolization, control of breathing, gas transfer and cardiovascular functions [46]. Therefore, it is reasonable to expect that these functions may be altered by increased levels of circulating catecholamines.

#### 4.2.2. Endocrine System

While there is a great deal of information on the effects of cocaine on the nervous system, the available data on the effects of cocaine on the endocrine system are not numerous and relate mainly to research in humans and mammals, as rats and monkeys. Most studies concern the hypothalamus-pituitary-adrenal (HPA) axis, which seems to be the main target of the action of cocaine; in our study, also the effects of cocaine on hypothalamus-pituitary-thyroid (HPT) axis, prolactin (PRL), and gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were studied.

- HPA axis

The HPA axis, also called stress axis, because involved in the stress response, is activated by the release of corticotropin-releasing-hormone (CRH). CRH is produced in one of the numerous nuclei of the hypothalamus, the paraventricular nucleus (PVN), although it is also present in other brain regions. CRH is released into the portal circulation of the median eminence, from which it reaches the pituitary corticotropic cells and induces the production of a polypeptide precursor, pro-opiomelanocortin (POMC). However, CRH is also a stimulator of growth hormone (GH) release and an inhibitor of LH release. The corticotropic cells of the pars distalis cut POMC into several end products as adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (α-, β-, and γ-MSH), β-lipotropin (LPH), β-endorphin and enkephalins, endogenous opioid peptides. However, POMC and related peptides, have been found also in many peripheral tissues, as skin and osteoarticular system [47]. ACTH, in turn, stimulates the fasciculata and reticularis areas of the adrenal cortex to release glucocorticoid hormones, basically cortisol in primates, along with a small amount of corticosterone, and corticosterone in rats. These hormones affect energy metabolism (1) inhibiting glucose utilization by peripheral tissues; (2) stimulating the liver to convert amino acids into glucose, and to store glucose in glycogen; (3) increasing mobilization of fat stores in non-neural tissues [48]. In turn, glucocorticoids regulate the activity of both corticotropic cells as well as PVN and other brain areas through a negative feed-back mechanism. In the brain, glucocorticoids bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), exerting effects through genomic and non-genomic mechanisms, that ultimately modify the behaviour and physiology of the organism [49].

Also, in fish there is a hypothalamus-pituitary-interrenal (HPI) axis, diversified between the different groups, and with anatomical and functional differences compared to mammals. Hypothalamic control of pituitary functions is mainly based on two regions that contain most neurosecretory neurons, the preoptic area (POA) and the hypothalamus proper, which in teleosts provide extensive innervation to adenohypophysis. Since in most teleosts there is a lack of a true median eminence and a portal system, the control of pituitary hormones is neuroglandular, by direct aminergic or peptidergic innervation [48]. In the Japanese eel, it has been observed that CRH has a high homology with that of mammals, and that CRH-immunoreactive (CRH-ir) fibers are very close and sometimes in contact with gonadotropin-releasing hormone immunoreactive (GnRH-ir) neurons, suggesting that CRH can regulate the activity of GnRH neurons [50]. In European eel, three crh paralogs genes were found (crh1a, crh1b, crh2), of which crh1b, mainly expressed in the brain, is considered the main regulator of corticotropic axis, whereas crh1a is mainly expressed in peripheral tissues as muscle and heart, and crh2 is weakly expressed in brain and peripheral tissues [51]. As in mammals, following CRH stimulation, in pituitary corticotropic cells, ACTH is obtained from a POMC precursor; ACTH, in turn, stimulates in teleosts the adrenocortical cells, present in the head kidney, to produce and release corticosteroids, mainly cortisol, and in lesser quantities corticosterone, aldosterone and some others. Cor-

tisol is the major corticosteroid, having many roles as regulation of sodium fluxes and gluconeogenesis, and response to stress [48].

Many bibliographical data indicate that cocaine affects HPA axis increasing the levels of plasma ACTH and cortisol/corticosterone in man, non-human primates and rodents, [52] and it is considered that also oxytocin (OXY) and arginine-vasopressin (AVP), together with CRH, are also involved in cocaine-induced ACTH release [53]. In eels, after a chronic exposure to cocaine, decreased levels of ACTH and corticosterone and high levels of cortisol were observed. After discontinuation of cocaine exposure, ACTH levels returned to normal, while corticosterone levels remained low and cortisol levels continued to be high. The increase in cortisol, induced by cocaine, is consistent with what has been observed in humans and mammals, whereas the decrease in ACTH levels could be explained with a negative feedback mechanism [43]. In fish, an increase in cortisol plasma levels is considered a marker of HPI axis activation and, therefore, of stress in the animals [54]. In the eels, the reproductive migration and gonadal maturation require the presence of adequate amounts of lipids as an energy source. Therefore, the increase in cortisol induced by cocaine could induce an excessive lipid use and pre-migratory energy deficit, with the possible consequence of delaying or preventing or blocking reproductive migration, as has already been suggested for many contaminants [55,56].

- HPT axis

The HPT axis is activated by the release of thyrotropin-releasing-hormone (TRH), a hypothalamic tripeptide hormone, produced by many nuclei but mainly in the PVN, and present also in other brain areas. TRH release is stimulated by NE secreting neurons and inhibited by DA secreting neurons, which both innervate the PVN. TRH is released into the portal circulation of the median eminence and stimulates pituitary thyrotropic cells of the adenohypophysis to release thyroid-stimulating hormone (TSH). It should be noted that while TRH stimulates TSH secretion, DA, and somatostatin (SST) inhibit it. TSH, in turn, stimulates the thyroid gland to synthesize and release the thyroid hormones, triiodothyronine ($T_3$) and tetraiodothyronine or thyroxine ($T_4$) and to increase iodide uptake by the thyroid cells. Normally, the circulating levels of $T_4$ are higher than levels of $T_3$, but $T_3$ has a higher biological activity, and most of $T_3$ is derived from peripheral $T_4$ deiodination. The thyroid hormones influence numerous processes as general metabolism, growth, differentiation, and reproduction; moreover, they regulate the synthesis of TRH and TSH through a mechanism of negative feedback [48].

Also in fish, an HPT axis exists, but with several differences than mammals, and between different groups of fish. For example, TRH in many species does not stimulate TSH release, and often the stimulating action of CRH on TSH release exceeds that of TRH. Moreover, TRH can induce prolactin (PRL), ACTH, α-MSH and growth hormone (GH) release. Thyroid hormones are $T_3$, the active form, and $T_4$, and in teleosts, they are essential for development, growth, and metamorphosis, and are also involved in osmoregulation, whereas their relationship with reproductive cycles may vary in different species. Moreover, thyroid hormones influence nutrient metabolism of carbohydrates, lipids, and proteins: in *A. anguilla* at glass stage, thyroid hormones decrease body protein content, and increase plasma glucose levels [57]. Unlike mammals, where thyroid hormones regulate the activity of the HPT axis through a negative feed-back on TRH and TSH release, it does not appear that in fish thyroid hormones can inhibit the release of TRH, but only of TSH at the pituitary level, as in the case of European eel [48,58]. Finally, numerous evidence shows that there is a close interaction between the HPI axis and the HPT axis in fish, and that pituitary and hypothalamic hormones of HPI and HPT axes may interact with each other, regulating the effects of thyroid hormones and cortisol. This evidence suggests that thyroid hormones, in addition to classical hormones, cortisol and adrenaline, also play an important role in responding to stress in fish [59]. Finally, in the European eel, thyroid hormones inhibit GH synthesis and release, acting directly at pituitary level [60].

There is little information about the effects of cocaine on the PT axis, which in rats [61] and humans [62] is not affected by chronic or acute cocaine administration; in humans

recent data indicate that cocaine use should be considered as a trigger for thyroid storm [63]. In our eels chronically exposed to cocaine, an increase in TSH levels and a decrease in $T_3$ levels were found, whereas $T_4$ levels appeared unchanged. After discontinuation of cocaine exposure, TSH values increased to normal levels, $T_3$ values increased but remained lower than control levels, and $T_4$ levels increased [43]. In the European eel, cortisol suppresses plasma thyroid hormone concentrations and increases plasma $T_3$, but not $T_4$ clearance [64], and in brook trout, cortisol stimulates the hepatic conversion of $T_4$ to $T_3$ [65]. In our experiment, increasing plasma cortisol levels, induced by cocaine exposure, is likely to lead to a reduction in $T_3$ levels, which in turn would increase TSH levels. We did not find a reduction in $T_4$ hormone levels, but the difference could be due to different exposure (chronic versus acute) and/or different dosage, or also to an increase of conversion of $T_4$ to $T_3$, that may be due to chronic, not acute, cocaine exposure.

- Prolactin

Prolactin (PRL) is produced in pituitary lactotrophs cells; the protein has a single chain of 199 amino acids, having many isoforms. The main PRL effects are associated with reproduction, growth, osmoregulation and integument, and the action of PRL often requires the interaction with other hormones. For example, glucocorticoids potentiate PRL actions, whereas progesterone inhibit PRL effects on the mammary gland competing for glucocorticoid binding sites and/or blocking gene activation by glucocorticoids. In lactating mammals, a neuroendocrine reflex triggered by teat stimulation induces PRL secretion, but many other factors regulate PRL release. An inhibitory control is provided by DA produced by neurons located in the arcuate nucleus of the hypothalamus; the regulation of the secretion of PRL also happens indirectly through many factors that act on the neurons DA stimulating or inhibiting them. For example, serotonin (5-HT), NE, histamine (H), somatostatin (SST) and many others inhibit DA neurons, stimulating PRL release, whereas TRH, acetylcholine (ACh), OXY, vasoactive intestinal polypeptide (VIP), and many others stimulate DA neurons, inhibiting prolactin release. Moreover, PRL itself feeds back on hypothalamic DA neurons regulating its own secretion [48,66].

In teleost, prolactin controls osmotic regulation in freshwater, regulating ion and water fluxes in urinary bladder, kidney, skin, gills, and intestine. Moreover, PRL stimulates mucus secretion by skin, intestine, and gills [67] and is involved in epithelial cell proliferation, together with cortisol [68].

In our experiment, after chronic cocaine exposure, the plasma PRL levels of eels were increased, but gradually decreased after discontinuation of cocaine exposure, to become lower than the control values ten days after cessation of exposure [69]. The increase in eel PRL levels agrees with the increase in PRL levels, which has been observed in rhesus monkeys after cocaine exposure [70]. In the eels, DA is a powerful PRL inhibitor [71]; therefore, the increase in plasma PRL level observed, despite the increase in cocaine-induced DA levels, can be explained by a change in dopaminergic regulation of PRL, as hypothesized in rhesus monkey [70].

- Gonadotropins

The gonadotropins, LH and FSH, are glycoproteins synthetized by the pituitary gland under the control of hypothalamic factors GnRH and gonadotropin-inhibiting hormone (GnIH), as well as activins and inhibins, produced by the gonads. LH induces in both sexes the synthesis of androgen hormones, and gamete release; FSH regulates in both sexes gamete development, and mainly in females, the conversion of androgens into estrogens by inducing the enzyme P450 aromatase [48].

Also, in fish LH and FSH, together with cortisol, are involved in the regulation of reproduction, in the development and maturation of gonads. In teleost, gametogenesis and the synthesis of androgens and estrogens is regulated by FSH, whereas final gamete maturation and release is regulated by LH, that also induces the production of a progesterone-like hormone [48]. In many teleosts, GnRH stimulates gonadotropins synthesis, whereas DA acts as an inhibitor. DA may also inhibit in some species the early steps of gametogenesis

and interact with GnRH in the control of puberty. In turn, sex steroids are part of a negative feedback circuit that affects DA synthesis and the expression of DA $D_2$ receptors, involved in DA inhibition of gonadotropins [72]. Moreover, in the European eel, cortisol stimulates LH synthesis [73].

In our experiment, after chronic cocaine exposure, eels had lower levels of LH and FSH, compared to control, not exposed eels [74]. These results are different from those obtained in humans [75], where gonadotropins increase, following acute cocaine administration, or in female rhesus monkeys, where increased LH levels in the presence of low basal E2 levels were found after cocaine administration [76]. Although the difference may be due to differences in the doses administered, the type of treatment (acute vs. chronic) or the different species considered, it is likely that the increase in DA levels had an important role. DA inhibits the release of gonadotropins, decreasing therefore FSH and LH levels. Cocaine also induced an increase in cortisol levels, that stimulates in the eel LH synthesis [76], but evidently the inhibitory effect of dopamine must have exceeded the stimulatory effect of cortisol.

### *4.3. Histopathological Changes Induced by Cocaine*

#### 4.3.1. Skin, Intestine, and Gills

In fish, skin, intestine, and gills are organs immediately exposed to water and its contaminants. In fact, they possess a thin epithelial layer that is characterized by a large surface area, which makes them particularly susceptible to aquatic contaminants [77]; moreover, skin, intestine and gills can accumulate cocaine [34]. The skin has a mechanical protection function and protects the animal from the invasion of chemical, physical or biological agents; moreover, skin also regulates osmotic exchanges between the body and the external environment. In the eels, the skin has a surface layer composed of stratified epithelium (epidermis) and a deep layer, of connective nature, separated by a thin basal lamina. The stratified epithelium has a deep stratum germinativum, a middle layer with many large club cells, marked by an apical vacuole, and a superficial layer with flattened epithelial cells and mucous cells (Figure 1). The club cells are involved in alarm reactions, as in their vacuoles are stored substances that are released into the water in dangerous situations and warn the conspecifics of the presence of a danger. The mucous cells are born in the germinative layer and migrate to the superficial one, where they release mucus, playing a key role in osmoregulation, defense, and social interactions. The turnover of mucous cells is continuous since they die when release mucus [23,78,79].

The intestine is involved in osmoregulation and feeding; in the eel, four layers can be observed: mucosa, submucosa, a muscular layer, and serosa. The mucosa has many irregular folds and consists of a simple cylindrical epithelium with caliciform mucous cells and absorbent enterocytes, and of a lamina propria of loose connective tissue. Under the submucosa, of connective nature, there are two muscular layers of smooth muscle, an inner, thick, circular layer, and an outer, thin, longitudinal layer. Finally, serosa has a connective nature too. However, the morphology of the alimentary tract varies in relation to the life cycle and metamorphic stage of eels, and at the silver stage, which precedes reproductive migration, when the eels stop feeding, the intestine shows signs of degeneration, as disappearance of folds, decrease in number of mucous cells, epithelial histolysis and thinning of the muscle layer (Figure 2) [23].

The gills play many roles in fish, as gas exchange, ionic and osmotic regulation, acid-base balance, and excretion of nitrogenous waste products. The gills are formed by a branchial filament delimited by a stratified epithelium in which flattened epithelial cells, mucous cells, chloride cells, neuroepithelial cells and undifferentiated cells are present. From each filament, perpendicular to it, originates a double row of secondary lamellae, which have externally flattened cells and, inside, undifferentiated cells. Below the epithelium are evident pillar cells, which separate the lamellar venous sinuses (Figure 3) [23].

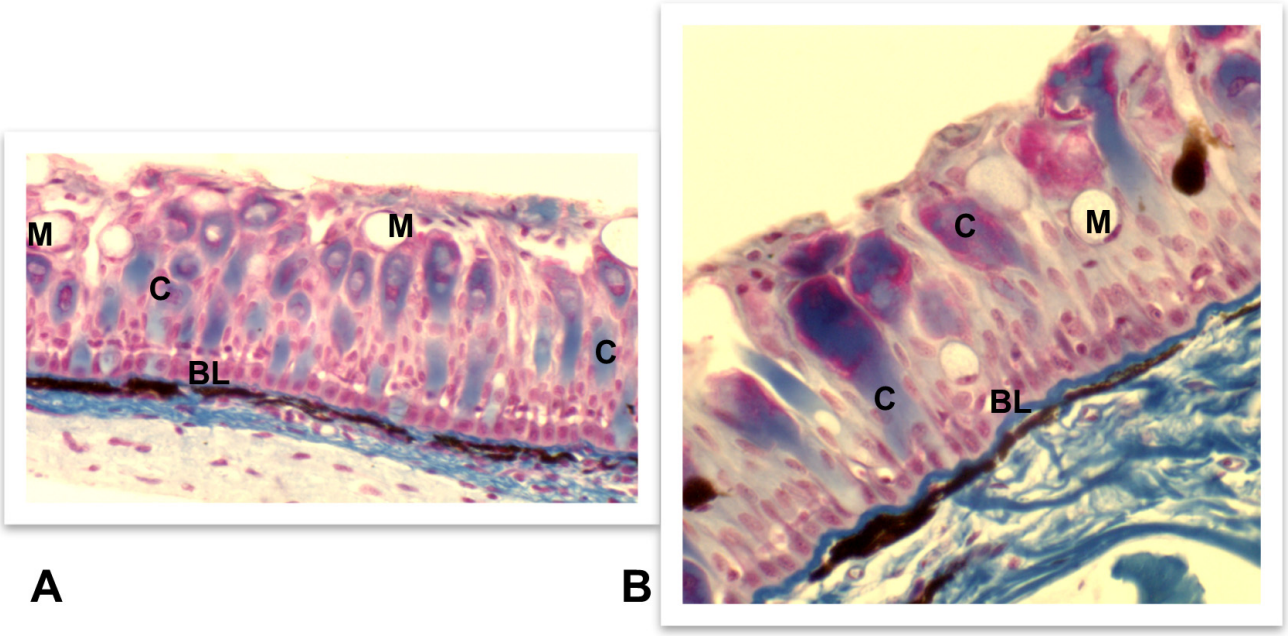


**Figure 1.** Light micrographs of the skin of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimen. Cocaine exposure (**B**) caused the thickening and folding of the basal lamina (BL), the loss of apical vacuoles in club cells (C) and the decrease of mucous cells (M). Magnification: 400×.

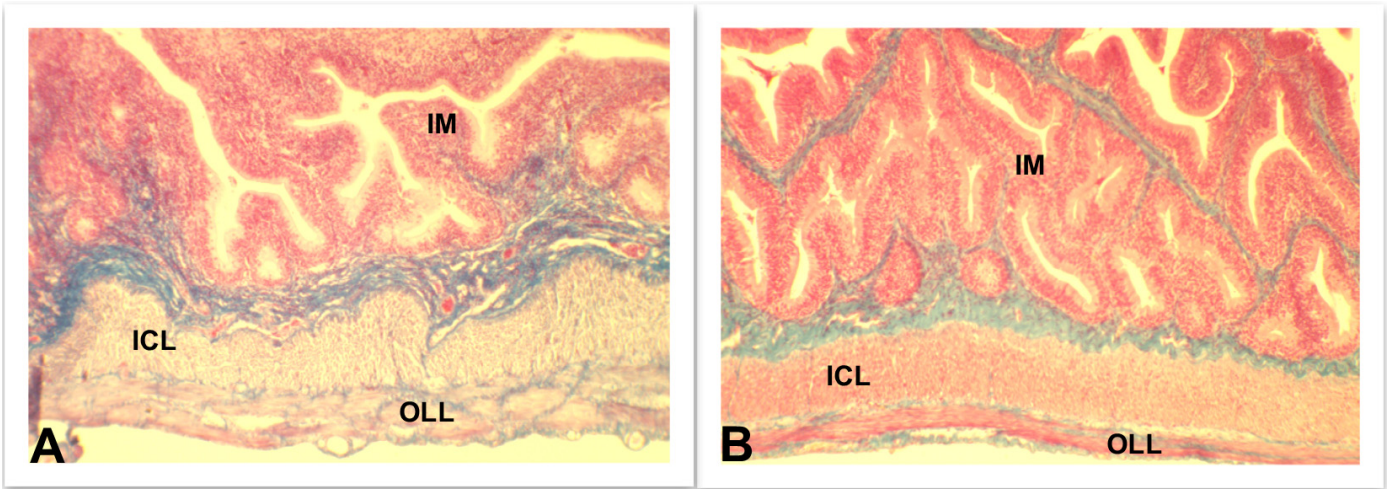


**Figure 2.** Light micrographs of the intestine of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimens. In control specimens, the intestinal mucosa (IM) showed signs of histolysis, whereas the muscular layers, inner circular layer (ICL) and outer longitudinal layer (OLL), showed sign of degeneration. In cocaine exposed specimens, the intestinal mucosa (IM) was well organized and had many folds, and the muscular layers appeared thick and well stained. Magnification: 100×.

The chronic exposure to cocaine induced similar changes in the epithelial tissue of skin, intestine, and gills [69,80]. The epidermis appeared thickened, with fewer small mucous cells, and club cells devoid of their vacuole, to indicate a massive release phenomenon; moreover, the underlying basal lamina appeared thickened and folded (Figure 1). Ten days after the interruption of cocaine exposure, mucous and club cells recovered normal morphology, while the basal lamina and the thickness of the epidermis were still different from the normal situation.

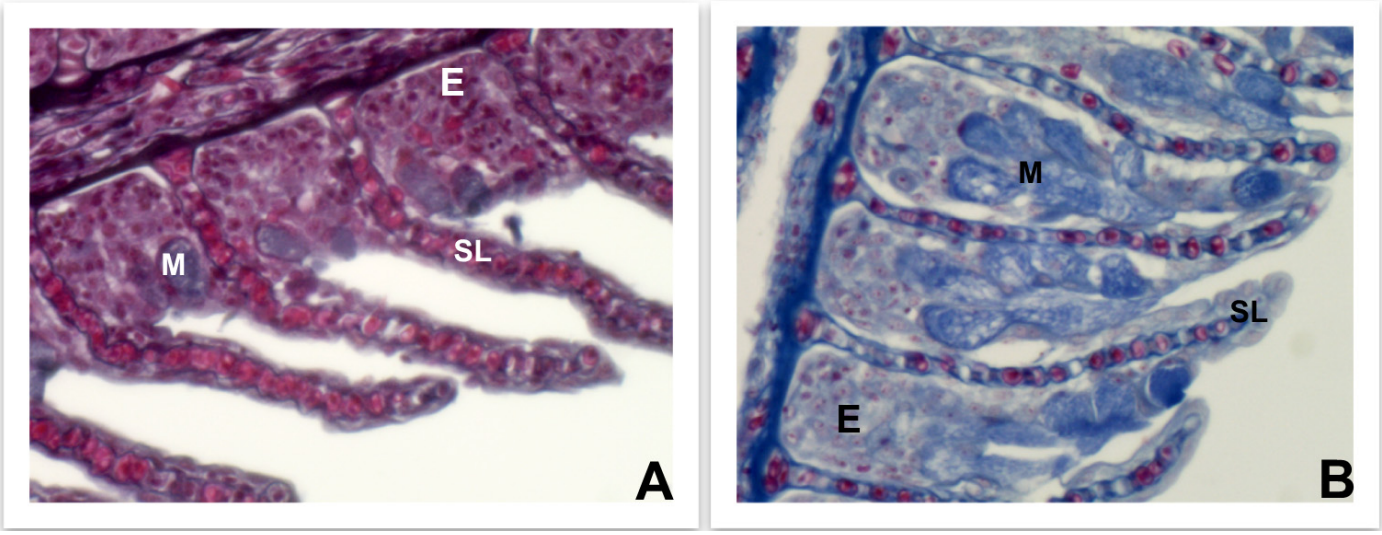


**Figure 3.** Light micrographs of the gills of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimens. In exposed specimens, the epithelium (E) appeared thickened, compared to control, and contained many mucous (M) cells, also present in the secondary lamellae (SL), which appeared partially fused. Magnification: 400×.

The intestinal epithelium, that at silver stage was degenerating, appeared thickened and convoluted, with many mucous cells. Also, the muscular layers were well organized, with fibers increased in size (Figure 2), that, however, began to regress ten days after the end of exposure to cocaine, when instead the epithelium still appeared well organized.

Finally, the gills showed a hyperplasic epithelium in which the mucous cells had increased a lot and appeared not only along the branchial filament and the interlamellar epithelium, but also in the secondary lamellae (Figure 3). Moreover, also partial, and total fusion of secondary lamellae was found; however, ten days after the end of exposure to cocaine, the appearance of the epithelium returned to normal [80]. Overall, the changes observed in skin, intestine, and gills, such as hyperplasia of the epithelium, the increase in the number of mucous cells, and the fusion of the secondary lamellae in the gills, are considered progressive changes, that suggest a defensive reaction against cocaine, and an adaptation of the eels to it [81]. These results differ from those obtained in humans and other species; for example, in humans and rats a systemic cocaine exposure causes skin oxidation, vasculitis, infectious complications and many other dermatologic conditions [82], and, in humans, gastrointestinal complications were seen following cocaine administration [83].

Finally, as regards the gills, data are available only in invertebrates where cocaine, at environmental concentrations, did not alter the oxidative status in the gills of *Mytilus galloprovincialis* [84] whereas, in *Perna perna*, mitochondrial DNA damage was seen in the gills, despite their detoxifying and antioxidant activity [85]. These differences may reflect differences in dosage and/or type of treatment, and species-specific sensitivity to cocaine; although the exact mechanism of action of cocaine on the skin, intestines and gills of eel needs to be clarified, it is likely that the effects of cocaine involve hormonal changes in cortisol and prolactin. Indeed, in fish the epithelial cell proliferation is controlled by cortisol and PRL; the latter also controls mucus secretion by skin, intestine and gills, differentiation, and proliferation of mucous cells [68] and proliferation of vascular smooth muscle cells [86]. Therefore, both hormones could be involved in the observed changes, although a direct action of cocaine cannot be ruled out.

#### 4.3.2. Skeletal Muscle

The skeletal muscle of European eel is composed by red and white fibers, the first located along the lateral line, the second forming the greatest volume of the body tissue. The diameter is greater in white fibers than in red ones, both fibers are surrounded by a

reticular connective tissue and have myofibrils regularly aligned and parallel each other in the sarcoplasm [23]. Following a chronic exposure to cocaine [27], the skeletal muscle had a significant cocaine content [34]. In addition, cocaine significantly damaged muscle fibers, causing lacerations and transverse ruptures (Figure 4), that were still present ten days after the cessation of cocaine exposure. Moreover, an increase in the mean diameter of fibers was observed, mainly in the red fibers, that appeared more damaged, showing signs of swelling, rarefaction of myofibrils and disorganization of the contractile apparatus The morphological changes were accompanied by increases (1) in the activity of cytochrome oxidase (COX), marker of oxidative metabolism and caspase 3, marker of apoptosis activation; (2) in the serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), biomarkers of skeletal muscle damage [87]. All these parameters were still altered ten days after the cessation of cocaine exposure. Cocaine is known to induce rhabdomyolysis, a syndrome characterized by the breakdown of muscle fibers and the release into the blood of cytosolic components, such as enzymes, electrolytes, and myoglobin. It is supposed that cocaine either acts directly on the muscle or through repeated ischemia caused by the vasoconstrictor activity of this drug. Subsequent reperfusion and generation of free radicals would damage sarcolemma, leading to the increase of cytosolic enzyme release [88]. It's possible that a similar mechanism also works in eels, since the characteristics of the muscle observed, and the high circulating values of the enzymes measured suggest the presence of rhabdomyolysis, induced by cocaine exposure.

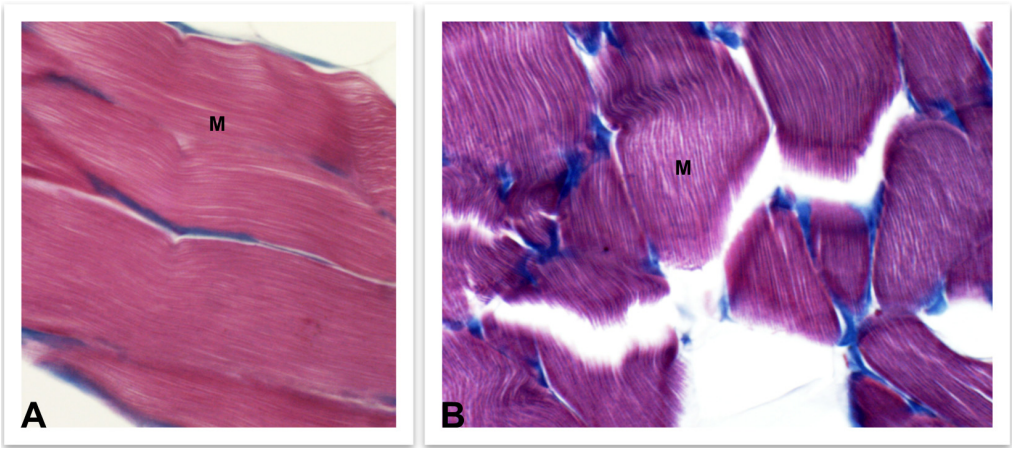


**Figure 4.** Light micrographs of the skeletal muscle of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimens. In exposed specimens, the muscle fibres (M) showed less compactness and numerous breaks. Magnification: 400×.

#### 4.3.3. Liver and Kidney

Liver and kidney are organs that play an important role in metabolization and excretion. In the eel, the liver parenchyma has anastomosing cords of polygonal hepatocytes, among which are present many sinusoids. At silver stage, the cytoplasm of hepatocytes contains large quantities of lipids (Figure 5).

Eel kidney has a pronephric portion with lymphoid and hematopoietic functions, and a posterior mesonephric kidney, with excretory and endocrine functions, where most nephrons are present. In the nephrons, a glomerulus within the Bowmans' capsule, proximal, distal, and collecting tubules are seen; moreover, kidney shows some melanomacrophage centers, containing melanin acting as scavenger for free radicals (Figure 6).

In both organs, cocaine induced numerous changes [89]. Liver and kidney showed signs of nuclear alterations, as kariolysis and pycnotic nuclei, and structural alterations, as necrotic areas, loss of cytoplasmic lipids and parenchymal cells in the liver; increase in the number of melanomacrophage centers (common in conditions of environmental stress), dilated renal tubules and reduced Bowman's space in the kidney. After the interruption to cocaine exposure, the liver showed signs of a gradual return to normal, while the

kidney was still very altered. The morphological alterations of liver and kidney were accompanied by increases (1) in the activity of cytochrome oxidase (COX), marker of oxidative metabolism and caspase 3, marker of apoptosis activation, in both organs; (2) in liver glucose-regulated protein (GRP)78 expression, a well-known regulator of apoptosis and a marker of endoplasmic reticulum (ER)-stress [90]; (3) in the level of blood glucose, a classical marker of stress response, whose increase is typical of the response of fish to pollutants; (4) in the serum levels of alanine aminotransferase (ALT), biomarker of liver injury, and C-reactive protein (CRP), marker of inflammatory process. Many of these parameters were still altered after the interruption of cocaine exposure.

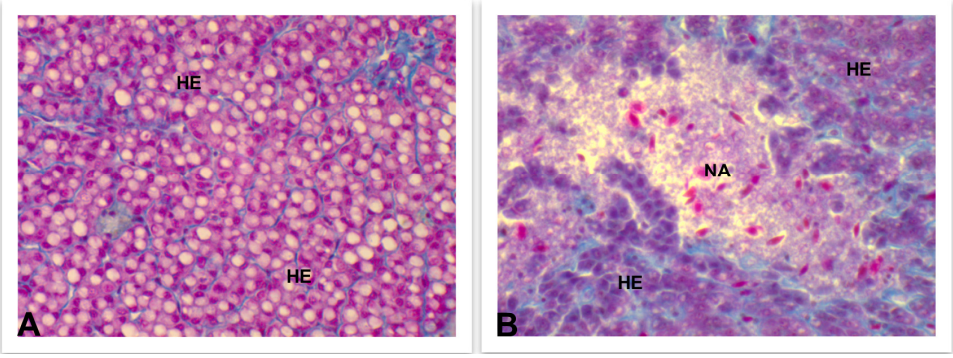


**Figure 5.** Light micrographs of the liver of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimens. In exposed specimens, the hepatocytes (HE) were devoid of lipids; necrotic areas (NA) and loss of parenchymal cells were evident. Magnification: 400×.

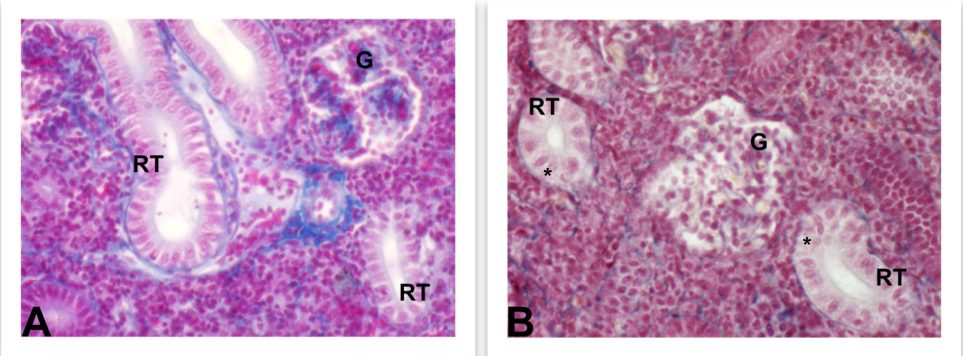


**Figure 6.** Light micrographs of the kidney of *Anguilla anguilla*. Mallory staining. (**A**) Control and (**B**) exposed specimens. In exposed specimens, the renal tubules (RT) showed nuclear alterations (*) and the structure of glomerulus (G) was disorganized. Magnification: 400×.

The damages observed in eel liver and kidney agree with the well-known hepatotoxicity and nephrotoxicity of cocaine when administered to humans, mammals, and fish [91]. Hepatotoxicity is believed to result from the production of oxygen reactive species (ROS) resulting from the metabolization of cocaine by hepatic esterases, while it is believed that nephrotoxicity may be partly associated with rhabdomyolysis, the release of large amounts of circulating myoglobin and its precipitation in the glomerular filtrate, resulting in damage to renal tubules. However, direct kidney damage caused by cocaine metabolization cannot be ruled out [92]. The increase in COX activity, in turn, might reflect the increase in specific mitochondrial enzyme activity and/or in mitochondrial protein mass, and could be related to an increased energy demand resulting from the metabolization activity of the liver and kidney. It has been observed that the hyperactivity of COX could activate the intrinsic pathway of apoptosis [93], and in fact, this hypothesis agrees with the increase of caspase 3 found in eels exposed to cocaine. Finally, the variations in all other parameters studied confirm that cocaine induced liver and kidney distress in eels.

#### 4.3.4. Ovaries

In the eel, gonads lie along the entire length of the body cavity and the eggs come out of the abdominal pore since female gonads have no separate outlet. European eel is an asynchronous, determinate, batch spawner [94]; at the silver stage, in the ovaries, it was possible to observe previtellogenic (pvOos), early vitellogenic (evOos) and fully vitellogenic (fvOos) oocytes, the latter having yolk vesicles throughout the cytoplasm. The oocytes were surrounded by follicular cells and connective cells such as theca endocrine cells. The exposure to cocaine [74] caused several changes, as the prevalence in the ovary of pvOos and connective tissue, and the presence of small size follicles, compared to control animals, although the number of follicles was nearly identical in both control and exposed animals. Moreover, cocaine modified the localization and reduced the intensity of antibody-labeled signal of three key enzymes playing a key role in oogenesis: P450 aromatase, catalyzing the conversion of androgens into estrogens and regulating sexual differentiation [95]; 3-β-hydroxysteroid dehydrogenase (3 β-HSD), regulating the synthesis of progesterone [96]; 17 β-hydroxysteroid dehydrogenase (17 β-HSD), involved in estrogen synthesis and gametogenesis [97]. These results agree with the adverse effects of cocaine on reproduction, observed in rabbits [98], rhesus monkeys [99] and *Drosophila melanogaster* [100]. Cocaine may act directly on the gonads and/or its action may be mediated through decreased FSH and LH levels; however, the exact mechanism of cocaine action requires further study.

## 5. Conclusions

European eel is an endangered species whose survival is hampered by many different factors, such as overfishing, habitat loss, pest attack and water contamination. Our studies have shown that the presence of illicit drugs in the water, as cocaine, may also be a problem for this species; indeed, the alterations in nervous and endocrine systems, and in peripheral tissues, induced by cocaine, could decrease its ability to survive and its reproductive fitness. Moreover, the presence of cocaine in the muscle, which is the edible part of the animal, can be a problem not only for the eel, which needs a healthy muscle to complete reproductive migration, but also for human consumption of this fish. In addition, in water contaminated by drugs, these are present not individually, but as mixtures, often accompanied by other types of pollutants; all this could make the effects on aquatic fauna, of which the European eel is a representative, unpredictable. Our study was performed on a single metamorphic stage, the silver stage, and at a single temperature, 15 °C, suitable for adequate consumption of energy resources and proper development of the gonads, and it is possible that the results may be different under different conditions. Moreover, although the study has been conducted on eels, it is possible to imagine that similar damage can be induced by cocaine, and probably by other drugs present in waters, also on other species of fish. Therefore, it is of great importance that governments adopt a strategy of upgrading wastewater treatment plants on the one hand, and that they undertake an appropriate environmental remediation policy on the other hand, to preserve aquatic fauna; indeed, as our results showed, after the interruption of cocaine exposure, many vital parameters of eels improved, or otherwise showed a tendency to improve.

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**Data Availability Statement:** The data presented in these studies are available on request from the corresponding author.

**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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